

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

**Positive-charged solid lipid nanoparticles as paclitaxel drug delivery system in glioblastoma treatment**

**This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/150103> since 2016-07-14T13:11:32Z

*Published version:*

DOI:10.1016/j.ejpb.2014.10.017

*Terms of use:*

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)



## UNIVERSITÀ DEGLI STUDI DI TORINO

This Accepted Author Manuscript (AAM) is copyrighted and published by Elsevier. It is posted here by agreement between Elsevier and the University of Turin. Changes resulting from the publishing process - such as editing, corrections, structural formatting, and other quality control mechanisms - may not be reflected in this version of the text. The definitive version of the text was subsequently published in EUROPEAN JOURNAL OF PHARMACEUTICS AND BIOPHARMACEUTICS, 88, 2014, 10.1016/j.ejpb.2014.10.017.

You may download, copy and otherwise use the AAM for non-commercial purposes provided that your license is limited by the following restrictions:

- (1) You may use this AAM for non-commercial purposes only under the terms of the CC-BY-NC-ND license.
- (2) The integrity of the work and identification of the author, copyright owner, and publisher must be preserved in any copy.
- (3) You must attribute this AAM in the following format: Creative Commons BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/deed.en>), 10.1016/j.ejpb.2014.10.017

The definitive version is available at:

<http://linkinghub.elsevier.com/retrieve/pii/S0939641114003166>

**Title:** Positive-charged solid lipid nanoparticles as paclitaxel drug delivery system in glioblastoma treatment.

**Author names and affiliations:** Daniela Chirio<sup>a</sup>, Marina Gallarate<sup>a</sup>, Elena Peira<sup>a</sup>, Luigi Battaglia<sup>a</sup>, Elisabetta Muntoni<sup>a</sup>, Chiara Riganti<sup>b</sup>, Elena Biasibetti<sup>c</sup>, Maria Teresa Capucchio<sup>c</sup>, Alberto Valazza<sup>c</sup>, Pierpaolo Panciani<sup>d</sup>, Michele Lanotte<sup>d</sup>, Laura Annovazzi<sup>e</sup>, Valentina Caldera<sup>e</sup>, Marta Mellai<sup>e</sup>, Gaetano Filice<sup>f</sup>, Silvia Corona<sup>f</sup>, Davide Schiffer<sup>e</sup>

<sup>a</sup> University of Turin, Dipartimento di Scienza e Tecnologia del Farmaco, via Giuria 9, 10125 Torino, Italy.

[daniela.chirio@unito.it](mailto:daniela.chirio@unito.it), [marina.gallarate@unito.it](mailto:marina.gallarate@unito.it), [elena.peira@unito.it](mailto:elena.peira@unito.it), [luigi.battaglia@unito.it](mailto:luigi.battaglia@unito.it), [elisabetta.muntoni@unito.it](mailto:elisabetta.muntoni@unito.it)

<sup>b</sup> University of Turin, Dipartimento di Oncologia, Regione Gonzole 10, 10043 Orbassano (TO), Italy.

[chiara.riganti@unito.it](mailto:chiara.riganti@unito.it)

<sup>c</sup> University of Turin, Dipartimento di Scienze Veterinarie, Largo Paolo Braccini 2, 10095 Grugliasco (TO), Italy.

[elena.biasibetti@unito.it](mailto:elena.biasibetti@unito.it), [mariateresa.capucchio@unito.it](mailto:mariateresa.capucchio@unito.it), [alberto.valazza@unito.it](mailto:alberto.valazza@unito.it)

<sup>d</sup> University of Turin, Dipartimento di Neuroscienze, Via Cherasco 15, 10126 Torino, Italy.

[vincy.bip@alice.it](mailto:vincy.bip@alice.it), [michele.lanotte@unito.it](mailto:michele.lanotte@unito.it)

<sup>e</sup> Policlinico di Monza, Centro di NeuroBioOncologia, Via Pietro Micca 29, 13100 Vercelli, Italy.

[davide.schiffer@unito.it](mailto:davide.schiffer@unito.it), [laura.annovazzi@cnbo.it](mailto:laura.annovazzi@cnbo.it), [valentina.caldera@cnbo.it](mailto:valentina.caldera@cnbo.it), [marta.mellai@cnbo.it](mailto:marta.mellai@cnbo.it)

<sup>f</sup> IRCCS Policlinico San Matteo, University of Pavia, Dipartimento di Malattie Infettive, V.le Golgi 19, 27100 Pavia, Italy,

[gaetano.filice@unipv.it](mailto:gaetano.filice@unipv.it)

27 Corresponding Author: Daniela Chirio, via P. Giuria 9, 10125 Torino, Italy

28 [daniela.chirio@unito.it](mailto:daniela.chirio@unito.it)

29 Phone: +39 0116707668 Fax: +39 0116707687

30

## 31 **Abstract**

32 Paclitaxel loaded solid lipid nanoparticles (SLN) of behenic acid were prepared with the  
33 coacervation technique. Generally, spherical shaped SLN with mean diameters in the range 300-600  
34 nm were obtained. The introduction of charged molecules, such as stearylamine and glycol chitosan  
35 into the formulation allowed to obtain positive SLN with Zeta potential in the 8-20 mV range and  
36 encapsulation efficiency in the 25-90% range.

37 Blood-brain barrier (BBB) permeability, tested in vitro through hCMEC/D3 cells monolayer,  
38 showed a significantly increase in the permeation of Coumarin-6, used as model drug, when  
39 vehicled in SLN. Positive-charged SLN do not seem to enhance permeation although stearylamine-  
40 positive SLN resulted the best permeable formulation after 24 h.

41 Cytotoxicity studies on NO3 glioblastoma cell line demonstrated the maintenance of cytotoxic  
42 activity of all paclitaxel-loaded SLN that was always unmodified or greater compared with free  
43 drug. No difference in cytotoxicity was noted between neutral and charged SLN.

44 Co-culture experiments with hCMEC/D3 and different glioblastoma cells evidenced that, when  
45 delivered in SLN, paclitaxel increased its cytotoxicity towards glioblastoma cells.

46

## 47 **Abbreviations:**

48 PTX, paclitaxel; SLN, solid lipid nanoparticles; BA, behenic acid; ST, stearylamine; GCS, glycol  
49 chitosan; BBB, blood-brain barrier; GBM, glioblastoma multiforme; CNS, central nervous system;  
50 NP, nanoparticles; cationic bovine serum albumin, CBSA; NHEJ, non-homologous end joining;  
51 PK, protein Kinase; CS, chitosan; Cou-6, coumarin 6; Na-BA, sodium behenate; CHOL,  
52 cholesterol; FD, freeze-dried; DMEM, Dulbecco's modified Eagle's medium; NS, neurosphere;

53 AC, adherent cell; ATM, ataxia telangiectasia mutated; ChK2, checkpoint kinase; p-53BP1, 53  
54 binding protein 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl-tetrazolium bromide; IF,  
55 immunofluorescence; HR, homologous recombination; TEM, transmission electron microscopy;  
56 EE, entrapment efficiency; Pgp, P-glycoprotein; MRP1, multidrug resistance related protein 1.

57

58 Keywords: paclitaxel, SLN, coacervation, BBB permeability, cytotoxicity, glioblastoma

59

60 Chemical compounds studied in this article:

61 Paclitaxel (PubChem CID: 36314)

62 Coumarin-6 (PubChem CID: 100334)

63 Behenic acid (PubChem CID: 8215)

## 64    **1. Introduction**

65    Glioblastoma multiforme (GBM) is the most common form of primary brain tumor in the central  
66    nervous system (CNS); its aggressive nature and evasiveness to treatments make it one of the most  
67    lethal cancers [1]. Current treatments for GBM provide a tumor surgical resection followed by  
68    pharmacotherapy and radiotherapy. Pharmacotherapy, directed by residual tumor cells elimination,  
69    ranges from common chemotherapeutic agents such as temozolomide to more recent anti-  
70    angiogenic agents and immunotherapeutic treatments [2]. However, anti-cancer therapeutic agents  
71    have not significantly increased the median survival of GBM patients over the past 10 years. The 5-  
72    year survival rate of GBM patients after treatment that includes surgical resection, radiation and  
73    chemotherapy, is 9.8%.

74    The failure of chemotherapy is due to the inability of intravenously administered anticancer agents  
75    to reach the brain parenchyma. An endothelial cell monolayer associated with pericytes and  
76    astrocytes, known as the blood–brain barrier (BBB), separates blood from the cerebral parenchyma  
77    and prevents the penetration of drugs into the CNS.

78    BBB is a functional unit composed by the peculiar endothelium of brain microvessels, the capillary  
79    basal lamina and the surrounding astrocytes, neurons, microglial cells and pericytes, which  
80    contribute to the maintenance of the barrier properties [3, Figure A.1]. The presence of tight  
81    junctions and adherent junctions between adjacent endothelial cells [4], the lack of fenestrations and  
82    pinocytotic vesicles [5], the abundance of efflux transporters belonging to the ATP binding cassette  
83    family on the endothelium luminal side [6] account for the low delivery of drugs, such as  
84    antineoplastic agents [7], from the bloodstream to the brain parenchyma.

85    Various invasive strategies have been developed to improve the penetration of drugs into the brain  
86    [8]. Traditional approach to overcome brain drug delivery obstacles includes direct intracerebral  
87    drug injection [9], which is associated with a high risk for the patient.

88    Less invasive strategies have also been investigated. One approach consists in generating a transient  
89    disruption of BBB in conjunction with the systemic administration of anticancer agents. The

90 intracarotid administration of a hyperosmotic solution such as mannitol led to a rapid diffusion of  
91 fluid across the cerebral endothelium, moving out of the endothelial cells into the vascular lumen  
92 and inducing the opening of the tight junctions for a few hours [10].

93 Another approach concerns the modification of drugs in order to make them more lipophilic,  
94 improving their penetration into the brain by passive diffusion. Lipophilic analogs and prodrugs  
95 were thus developed [11].

96 A more recent strategy to deliver drugs to the brain is the use of colloidal polymers to form  
97 nanometer sized carriers [12]. The basic reason of common acceptance of these vehicles is due to  
98 their drug release controlled profile of as well as to their selected targeting mechanism. Targeting  
99 action may be due to the steric hindrance created by nano-vectors: after parenteral administration,  
100 due to steric phenomenon they conceal themselves from opsonization event induced by tissue  
101 macrophages. By this way they achieve targeting ability to the brain and partially avoid other  
102 reticuloendothelial system organs like liver, spleen, etc [13].

103 From the last few decades, nanoparticles (NP) have attracted considerable interest in targeting drug  
104 molecules to the brain [14]. The correct mechanism of barrier opening by NP is not exactly known;  
105 the delivered NP enter into the brain by crossing the BBB by various endocytotic mechanisms, as  
106 polymeric albumin or poly(butylcyanoacrylate) NP are reported to enter into the brain by their small  
107 size mediated endocytosis [15,16]. An increased drug retention in brain blood capillaries combined  
108 with an adsorption to capillary can increase drug transport due to an enhanced concentration  
109 gradient; an increase of BBB fluidization, an opening of tight junctions between endothelial, an  
110 inhibition of the P-glycoprotein efflux system are other possible mechanisms that can increase brain  
111 drug concentration [17].

112 Although NP may be designed to entrap high molecular weight or hydrophilic therapeutics, BBB  
113 retardation of drug NP entrapped is based on NP characteristics and not on the therapeutic agent  
114 [18].

115 Indeed, poly(butyl cyanoacrylate) NP overcoated with 1% polysorbate 80 have been experimentally  
116 successful as brain drug delivery for doxorubicin [19] and dalargin [20], poly(lactic-co-glycolic  
117 acid) and cetyl alcohol/polysorbate NP for paclitaxel brain delivery [21,22].

118 In addition to BBB functional characteristics limiting permeation, brain microvasculature  
119 endothelia also present a luminal electrostatic barrier at physiologic pH. The negative electrostatic  
120 charge is created by surface expression and adhesion of the glycocalyx residues: proteoglycans,  
121 sulfated mucopolysaccharides, and sulfated and sialic acid-containing glycoproteins and glycolipids  
122 [23]. Cationic molecules have been shown to occupy anionic areas at the BBB endothelium [24]  
123 and increase BBB permeability via a presumed tight junction disruption [25].

124 Recent *in vitro* reports have demonstrated that positive-charged NP have an increased brain  
125 distribution compared to anionic and neutral NP [26]. However, there is little data regarding brain  
126 permeability of positive-charged NP. Lu et al. [27] developed and evaluated cationic bovine serum  
127 albumin (CBSA) conjugated with poly(ethyleneglycol)–poly(lactide) NP (CBSA–NP). To evaluate  
128 the effects of brain delivery, BSA conjugated with pegylated NP (BSA–NP) was used as the control  
129 group and Coumarin-6 was incorporated into the NP as the fluorescent probe. The qualitative and  
130 quantitative results of CBSA–NP uptake experiment compared with those of BSA–NP showed that  
131 rat brain capillary endothelial cells took in much more CBSA–NP than BSA–NP at 37 °C, at  
132 different concentrations and time incubations. After a dose of 60 mg/kg CBSA–NP or BSA–NP  
133 injection in mice caudal vein, fluorescent microscopy of brain coronal sections showed a higher  
134 accumulation of CBSA–NP in the lateral ventricle, third ventricle and periventricular region than  
135 that of BSA–NP. In an experimental work Lockman et al. [15] evaluated the effect of neutral,  
136 anionic and cationic charged NP on BBB integrity and NP brain permeability. Neutral NP and low  
137 concentrations of anionic NP had no effect on BBB integrity, whereas, high concentrations of  
138 anionic NP and cationic NP disrupted the BBB. The brain uptake rates of anionic NP at lower  
139 concentrations were higher than of neutral or cationic formulations at the same concentrations.



140 In literature, many authors studied solid lipid nanoparticles (SLN) as drug delivery systems to  
141 deliver drugs to the CNS [28,29]. SLN are disperse systems having size ranging from 1 to 1000 nm  
142 which represent an alternative to polymeric particulate carriers and are composed of physiological  
143 or biocompatible lipids or lipid molecules with a history of safe use in therapy and are generally  
144 suitable for intravenous administration.

145 As few data are present in literature about positive-charged SLN for brain delivery, the purpose of  
146 this work will be to prepare, characterize and evaluate in vitro the potential of positive-charged SLN  
147 to vehicle paclitaxel (PTX) to the brain for GBM treatment.

148 PTX, a diterpene isolated from *Taxus brevifolia*, is one of the most active chemotherapeutic agents  
149 against a wide panel of solid tumors including urothelial, breast, lung, and ovarian cancers. It has  
150 been demonstrated that PTX is effective against glioblastoma cells *in vitro* [30,31], however its  
151 clinical use is limited due to its poor BBB penetration capability and drug-resistance [32,33].

152 Due to its low water solubility, PTX is formulated in a mixture of Cremophor® EL and dehydrated  
153 ethanol (50:50 v/v) a combination known as Taxol®. However, Taxol® has some severe side  
154 effects related to Cremophor® EL and ethanol [34]. Therefore, there is an urgent need for the  
155 development of alternative PTX formulations.

156 Recently, a new solvent-free technique, defined as “coacervation”, was developed to prepare fatty  
157 acids-based SLN [35]. Briefly, a fatty acid alkaline salt micellar solution in the presence of an  
158 appropriate polymeric stabilizer was prepared; when the pH is lowered by acidification, the fatty  
159 acid precipitates as nanoparticles owing to proton exchange between the acid solution and the  
160 sodium salt.

161 SLN were prepared using two different positive-charged substances: ST or GCS. ST possess a  
162 hydrocarbonic chain that can probably be incorporated within the lipid matrix, while its positive  
163 charge is exposed to the external surface. GCS, a chitosan derivative conjugated with ethylene  
164 glycol branches, is a water soluble at a neutral/acidic pH values polymer whose pendant glycol  
165 branches increase both the aqueous solubility of the native chitosan (CS) and provide steric

166 stabilization [36]. Mao et al [37] have established that CS is capable of opening the tight junctions  
167 of epithelial cells and it can improve the uptake of hydrophilic drugs including peptides.  
168 For the above mentioned purpose, PTX loaded cationic SLN were prepared and extensively  
169 characterized *in vitro* with regard to their physicochemical properties, their capacity to load and  
170 release PTX, their cytotoxicity and their permeability across hCMEC/D3 cell lines taken as an *in*  
171 *vitro* model of BBB.

## 172 2. Experimental

### 173 2.1 Chemicals

174 Sodium Behenate (Na-BA) was purchased from Nu-Chek Prep, Inc. (Elysian, U.S.A.), Tween<sup>®</sup>80,  
175 sodium dodecyl sulfate, Triton X-100 and cholesterol from Fluka (Buchs, Switzerland), paclitaxel  
176 (PTX) from Indena (Milan, Italy), citric acid from A.C.E.F. (Fiorenzuola d'Arda, Italy), 80%  
177 hydrolyzed PVA 9000–10000 Mw (PVA 9000), Pluronic F68, stearylamine (ST), glycol chitosan  
178 (GCS), coumarin 6 (Cou-6), triethanolamine phosphate, trehalose, penicilin-streptomycin,  
179 hydrocortisone, ascorbic acid, Hepes and b-FGF from Sigma (Dorset, UK), hydrochloric acid,  
180 sodium hydroxide and sodium phosphate monobasic from Merck (Darmstadt, Germany),  
181 Cremophor<sup>®</sup> EL from BASF (Ludwigshafen am Rhein, Germany), EBM-2 basal medium from  
182 Lonza, (Basel, Switzerland), fetal bovine serum Gold from PAA The Cell Culture Company  
183 (Pasching, Austria), rat collagen-I from Trevigen (Gaithersburg, Maryland, USA), chemically  
184 defined lipid concentrate from Invitrogen Life technologies (Carlsbad, California, USA), methanol  
185 and ethanol from Carlo Erba (Val De Reuil, France). Deionized water was obtained by a MilliQ  
186 system (Millipore, Bedford, MO).

187

### 188 2.2 hCMEC/D3 cells

189 hCMEC/D3 cells, a primary human brain microvascular endothelial cell line that retains the  
190 property of BBB *in vitro*, were cultured as reported [38]. Briefly, cells were maintained in Petri  
191 dishes coated with 150 µg/ml rat collagen-I and cultured in EBM-2 basal medium supplemented  
192 with 5% v/v fetal bovine serum Gold, 1% v/v penicilin-streptomycin, 1.4 µM hydrocortisone, 5  
193 µg/ml ascorbic acid, 10 mM Hepes, 1% v/v Chemically Defined Lipid Concentrate, 1 ng/ml b-  
194 FGF. In all the experiments cells were used between passages 29 and 34. For the permeability  
195 assays, cells were seeded at 50,000/cm<sup>2</sup> and grown for 7 days up to confluence in Petri dishes and  
196 Transwell devices (0.4 µm diameter pores-size, Corning Life Sciences, Chorges, France), with 1.5

197 ml/cm<sup>2</sup> and 3 ml/cm<sup>2</sup> of the culture medium described above in the Transwell insert and in the  
198 lower chamber, respectively.

199 Before each experiment we measured the transendothelial electrochemical resistance (TEER) and  
200 the permeability coefficient of dextran-FITC, [<sup>14</sup>C]-sucrose and [<sup>14</sup>C]-inulin, taken as parameters  
201 of paracellular transport across hCMEC/D3 monolayer, as described in [39]. TEER value was  
202 between 30 and 40 Ω x cm<sup>2</sup>, dextran-FITC permeability coefficient was  $0.015 \pm 0.003 \times 10^{-3}$   
203 cm/min, [<sup>14</sup>C]-sucrose permeability coefficient was  $1.19 \pm 0.21 \times 10^{-3}$  cm/min, [<sup>14</sup>C]-inulin  
204 permeability coefficient was  $0.56 \pm 0.09 \times 10^{-3}$  cm/min. These values suggested the functional  
205 integrity of BBB monolayer [38].

206

### 207 *2.3 GBM cell lines and culture conditions*

208 Primary human GBM cell lines NO3 and CV17 were obtained from primary human GBMs  
209 surgically resected at the Department of Neuroscience, Neurosurgical Unit, University of Turin,  
210 Italy. The histological diagnosis was performed according to World Health Organization (WHO)  
211 guidelines. The study was in compliance with the local institutional review board and Committee on  
212 Human Research and with the ethical human-subject standards of the World Medical Association  
213 Declaration of Helsinki Research. Written informed consent was obtained from all patients.

214 010627 GBM cell line was a kind gift of Dr. Galli R, DIBIT San Raffaele, Milan, Italy). For  
215 cytotoxicity studies in co-culture models U87-MG cells (ATCC, Rockville, MD) were also used.

216 By neurosphere assay, from a resected tumor tissue we obtained neurosphere (NS) or adherent cell  
217 (AC) or both cell types [40]. NS lines were cultured on Dulbecco's modified Eagle's medium  
218 (DMEM)/F-12 supplemented with 20 ng/ml epidermal growth factor (EGF) and 10 ng/ml basic  
219 fibroblast growth factor (bFGF); AC lines were grown on DMEM supplemented with 10% fetal  
220 bovine serum (FBS) for AC. Both cultures were maintained at 37°C and at 5% O<sub>2</sub>/CO<sub>2</sub> in a  
221 humidified multigas Sanyo MCO-18M incubator (Sanyo Scientific, Bensenville, IL).

222 All cell cultures were periodically checked for *Mycoplasma* contamination (e-Myco™  
223 Mycoplasma PCR Detection kit, iNtRON Biotechnology, Korea).

224

#### 225 2.4 PTX-loaded SLN preparation

226 Different SLN formulations were prepared (Table 1); non-charged SLN were prepared as reference  
227 to evaluate the positive-charge influence.

228 Non-charged SLN: SLN were prepared by the coacervation method [35]. Briefly, appropriate  
229 amounts of Na-BA and PVA 9000 were dispersed in 5 ml deionized water and the mixture was then  
230 heated under 5 min-stirring (300 rpm) just above the Krafft point of Na-BA (75 °C) to obtain a clear  
231 solution (micellar solution). A selected acidifying solution (100 µl 1M NaH<sub>2</sub>PO<sub>4</sub> + 160 µl 1M HCl)  
232 was then added drop-wise until pH 4.0 was reached. The obtained BA SLN suspension was then  
233 cooled in a water bath under 10 min-stirring at 300 rpm until 15 °C temperature was reached. SLN  
234 suspension was then again heated under stirring (300 rpm) just above the melting point of BA (80  
235 °C). Different amounts of 30 mg/ml ethanol PTX solution, heated at the same temperature of SLN  
236 suspension, were added to the warm mixture and then the sample was again cooled in a water bath  
237 under stirring at 300 rpm until 15 °C was reached.

238 Charged SLN: SLN were prepared as described for non-charged SLN, introducing the positive-  
239 charged agent as follows:

- 240 - ST-SLN: a fixed amount of 30 mg/ml 2-propanol ST solution (Table 1) was added to the warm  
241 aqueous Na-BA solution just above the Krafft point of Na-BA.
- 242 - GCS-SLN: an appropriate amount of GCS (Table 1) was added at the beginning of the  
243 preparation before obtaining the micellar solution.

244 All SLN formulations were also prepared in the presence of cholesterol (CHOL-SLN): 100 or 150  
245 µl of 5 mg/ml ethanol CHOL solution were added to the micellar solution immediately before the  
246 addition of the acidifying solution.

247 All SLN formulations were also prepared without drug (unloaded SLN) adding the same amount of  
248 ethanol instead PTX-ethanolic solution.

249 Fluorescent SLN to be employed in *in vitro* experiments were prepared using Cou-6 as fluorescent  
250 lipophilic model drug (Cou-6-CHOL-SLN). 5 ml SLN were prepared in aseptic conditions (under  
251 laminar flow airfilter starting from materials sterilized with UV or in autoclave) adding 100  $\mu$ l  
252 2mg/ml Cou-6 ethanol solution after SLN fusion. The samples were then cooled in a water bath  
253 under stirring at 300 rpm until 15°C temperature was reached. SLN were lyophilized using a  
254 programmable freeze-dryer (Shin PVTFD10R, Shinil Lab, Korea) without cryoprotectant addition.  
255 Slow freezing was carried out on the shelves in the freeze dryer (shelf temperature -40 °C).  
256 Samples were lyophilized for 24 h from -40 °C to 25 °C at a 5 °C/h. increasing rate. Lyophilized  
257 products were reconstituted by magnetic stirring adding the same water amount.

258

## 259 2.5 SLN characterization

260 SLN shape and mean sizes were characterized by Transmission electron microscope (TEM, CM 10  
261 Philips, The Netherlands) spraying the SLN suspension on the microscope grid by means of an  
262 aerosol-sampling device.

263 Cou-6 localization into SLN dispersion was determined using optical microscopy equipped with a  
264 fluorescent lamp (Leica DM 2500, Solms, Germany) at 1000x magnification.

265 SLN particle sizes, polydispersity indexes (PDI) and Zeta potential were determined one hour after  
266 preparation using laser light scattering technique-LLS (Brookhaven, New York, USA). Size  
267 measurements were obtained at an angle of 90° at 25 °C using the number method. The dispersions  
268 were diluted with water for size determination or with 0.01 M KCl for Zeta-potential determination,  
269 in order to achieve the prescribed conductivity. Size measurements were also recorded diluting  
270 samples with grown medium used to culture hCMEC/D3 cells to mime the conditions under which  
271 SLN undergo *in vitro* experiments. For stability studies, the samples were stored at 4°C. All data  
272 were determined in triplicate.

PTX entrapment efficiency (EE%) was calculated as the ratio between PTX amount in SLN and that in the starting micellar solution  $\times 100$ . PTX EE% determination was performed as follows: 1 ml SLN suspension was centrifuged for 15 min at 62,084 g, the precipitate was washed twice with 1 ml ethanol:water 30:70 to eliminate adsorbed PTX. The solid residue was dissolved in 1 ml ethanol, 0.5 ml water were then added to precipitate the lipid matrix and the supernatant obtained was injected in HPLC for PTX quantification.

EE% was also determined after 1:100 dilution in different media to mime the dilution that SLN can undergo after administration. Briefly, 500  $\mu$ l SLN were introduced in a 50 ml flask and 49.5 ml water or 0.1 M phosphate buffer pH 7.4 or 0.1 M citric acid buffer pH 5.5 were added. The suspension was stirred for 2 hours. 8 ml suspension were centrifuged for 15 min at 62,084 g and then treated as previously described.

284

## 2.6 HPLC analysis

HPLC analysis was performed using a LC9 pump (Shimadzu, Tokyo, Japan) with a Chromosystem™ ODS 2.5  $\mu$  125 $\times$ 4.6mm column and a C-R5A integrator (Shimadzu, Tokyo, Japan); mobile phase: CH<sub>3</sub>CN:H<sub>2</sub>O 60:40 (flow rate 1ml min<sup>-1</sup>); detector: UV  $\lambda$ =227 nm (Shimadzu, Tokyo, Japan). Retention time was 2.6 min.

The limit of quantification, defined as the lowest PTX concentration in the curve that can be measured routinely with acceptable precision and accuracy, was 0.013  $\mu$ mol/ml; the limit of determination, defined as the lowest detection limit, was 0.005  $\mu$ mol/ml (signal to noise>2.0).

293

## 2.7 SLN and PTX stability

SLN sizes and PTX EE% of samples stored at 4 °C were monitored for 90 days to study SLN physical stability and PTX chemical stability. Mean sizes were determined by LLS and PTX EE% over time was obtained as described by HPLC.

298 The same SLN suspensions were freeze-dried without adding any cryoprotectant (FD-SLN) using a  
299 Modulyo Freeze Dryer (Edwards Alto Vuoto, Italy). The resulting samples, rehydrated with the  
300 same water amount, were also characterized by size, Zeta potential and EE% determination.

301

## 302 2.8 PTX *in vitro* release

303 *In vitro* release of PTX was determined using the non-equilibrium dialysis method [41]. A  
304 multicompartimental rotating cell system consisting of donor and receptor compartments of equal  
305 volume (1.5 ml) separated by a dialysis membrane (cut-off 12,000 Da) was used. Receiving  
306 medium was 0.1 M phosphate buffer (pH 7.4) containing 0.1% v/v Tween®80. Solution of PTX in  
307 receiving medium and SLN suspensions were used as donor formulations. At fixed times, the  
308 receptor solution was tipped out and used for HPLC analysis and the cell was refilled with fresh  
309 receiving medium. Drug concentration was determined by HPLC. The results were evaluated as  
310 PTX apparent permeability constant ( $K_{d_{app}}$  [cm h<sup>-1</sup>]) calculated from the slope of the straight line  
311 obtained by plotting the amount of PTX diffused from the donor formulation versus time, assuming  
312 pseudo zero-order kinetics.

313

## 314 2.9 Permeability of SLN through hCMEC/D3 cell monolayer

315 hCMEC/D3 cells, seeded as reported above in Transwell devices, were incubated at day 7 with  
316 SLN 2, SLN 9, SLN 15 loaded with Cou-6, for 0.5, 3 and 24 h in the Transwell  
317 insert. The amount of SLN added was 100 µl diluted with 900 µl of medium. This dilution of  
318 SLN was the highest concentration devoid of cytotoxic effects on hCMEC/D3 cells, as previously  
319 reported [42]. In parallel, a set of Transwell inserts were incubated with Cou-6 aqueous suspension  
320 obtained with PVA 9000 as suspending agent, containing the same concentration of Cou-6  
321 present in SLN. At the end of the incubation time, the medium in lower chamber was collected,  
322 diluted 1: 20 into ethanol, sonicated with 10 bursts of 1 s to disrupt SLN, and centrifuged at 62,084  
323 g for 5 min, to pellet debris. The dilution of the medium was necessary to obtain a fluorescence



324 falling in the detection range of the LS-5 spectrofluorimeter (PerkinElmer, Waltham, MA), used to  
325 measure the fluorescence of Cou-6.

326 Excitation and emission wavelengths were  $485 \pm 20$  nm and  $528 \pm 20$  nm, respectively. The  
327 fluorescence of Cou-6 at  $t_0$  in the upper chamber, was considered 100%. The fluorescence of Cou-6  
328 measured in the medium collected from the lower chamber was expressed as percentage of  
329 fluorescence in the upper chamber at  $t_0$ . The fluorescence of the medium without Cou-6 was  
330 considered as blank and was subtracted from the fluorescence of all the other experimental  
331 conditions.

332 To measure the amount of Cou-6 adsorbed on the Transwell membrane, Cou-6, either free or loaded  
333 in SLN, was added in empty Transwells in the same experimental conditions of the Transwells  
334 containing hCMEC/D3 cells, then the medium from the insert and the lower chamber was removed;  
335 the Transwell membrane was washed with PBS and 0.5 ml ethanol was added to solubilize the  
336 Cou-6 adsorbed on the membrane. The solution was collected and its fluorescence was read as  
337 reported above. The fluorescence was always less than 4% of the fluorescence measured in the  
338 lower chamber medium in all the experimental conditions, suggesting that such very low absorption  
339 of Cou-6 on the Transwell insert did not influence the results of the permeability assays.

340 The level of significance was determined by a Student's  $t$  test. Statistical significance was defined  
341 as  $p < 0.001$ .

342

#### 343 *2.10 In vitro cytotoxicity assay*

344 For cytotoxicity studies, NO3 cells (both NS and AC) and CV17 NS were used at various passage  
345 numbers between 20 and 30, whereas 010627 cells (both NS and AC) were used at passage  
346 numbers between 160 and 180.

347 Free PTX (Sigma Aldrich Co., St. Louis, MO, USA) was dissolved in 100% DMSO for stock  
348 solutions and dilutions for cell treatments were made extemporaneously in culture medium, so that  
349 the final concentration of DMSO never exceeded 0.3% (v/v).

Cell lines were treated for 72 h with increasing doses (20, 100, 500, 1400 nM) of free PTX.

After exposure the cytotoxic effect of free Paclitaxel (PTX) and PTX-loaded SLN against tumor cells was evaluated assessing the number of viable cells by the Trypan blue dye exclusion test, using a TC20 automated cell counter (Bio-Rad, Berkeley, CA, USA). As for AC, results were confirmed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Roche, Diagnostic Corporation Indianapolis, IN, USA) [43].

For Trypan blue assay, cells were plated at a density of  $200 \times 10^3$  cells in 5 ml medium in a  $25 \text{ cm}^2$  flask and treated with PTX (free in solution or encapsulated in SLN2, SLN9 or SLN15) at  $37^\circ\text{C}$  in 5%  $\text{O}_2/\text{CO}_2$  for different times (24 and 72 h). Stock solutions were diluted extemporaneously in fresh medium at the desired concentration and administered to cells.

Cytotoxicity was expressed as number of surviving cells as percentage of control (untreated cells).

For MTT assay, cells were seeded in 96-well plates ( $1 \times 10^4$  cells/well in 100  $\mu\text{l}$  medium) and treated with the different concentrations and times above indicated of the drug (free or encapsulated) at  $37^\circ\text{C}$  in 5%  $\text{O}_2/\text{CO}_2$ . The medium with the SLN was replaced with fresh medium and 10  $\mu\text{l}$  of MTT solution (5mg/ml) were added to each well and incubated for 4 h at  $37^\circ\text{C}$ . The extent of cell viability is indicated by mitochondrial conversion of yellow MTT, a tetrazole, to purple formazan by the living cells. Formazan crystals were solubilized overnight in 10% sodium dodecyl sulfate (SDS), 0.01 M HCl and optical density was measured at 570 nm (test wavelength) and 660 nm (reference wavelength) using a microplate spectrophotometer (Synergy HT, BioTek Instruments Inc., Winooski, VT, USA). Cell viability was expressed as a percentage of the absorbance measured in the treated cells compared to the control (untreated cells). All experiments were performed in quadruplicate.

The concentration of free PTX which caused a 50% cell growth inhibition ( $\text{IC}_{50}$ ) compared with untreated controls, was calculated by non-linear regression for each cell line.

To investigate the cytotoxic effect of PTX incorporated in SLN, a drug concentration of 100 nM was chosen for the following *in vitro* experiments.

376 Cell viability was also measured after treatment with unloaded SLN (SLN2, SLN9 and SLN15) at  
377 the highest SLN concentration used for the study, *i.e.* 0.1% w/v.

378 The level of significance was determined by a two-tailed Student's *t* test. Statistical significance  
379 was defined as  $p < 0.05$  or  $p < 0.01$ .

380

#### 381 *2.11 DNA damage/repair study by Immunofluorescence (IF)*

382 The occurrence of a DNA damage after SLN treatment was investigated through the analysis by IF  
383 of  $\gamma$ -H2AX histone, that localizes at sites of DNA fragmentation as subnuclear foci.

384 The cell response to DNA insults was studied monitoring the activation of p-ATM, p-Chk2 and p-  
385 53BP1. HR or NHEJ repair system activities were analyzed through RAD51 or Ku70/Ku80 and  
386 DNA-PKcs proteins respectively. After 72 h treatment with unloaded SLN (SLN9) or with 100 nM  
387 PTX-loaded SLN9, cells were fixed for 20 minutes with 4% paraformaldehyde at room  
388 temperature, rinsed three times with PBS, blocked/permeabilized with PBS containing 2% of the  
389 appropriate serum and 0.1% Triton X-100 for 30 minutes and stained with the following primary  
390 antibodies: mouse anti- $\gamma$ -H2AX (Ser139), mouse anti-p-ATM (Ser1981) (both from Millipore),  
391 rabbit anti-p-Chk2 (Thr68) (Cell Signaling Technology), rabbit anti-p-53BP1 (Ser25) (Bethyl  
392 Laboratories), mouse anti-RAD51, mouse anti-DNA-PKcs and mouse anti-Ku70/Ku80 (all from  
393 NeoMarkers). Negative controls were obtained by omitting the primary antibody. Alexa Fluor®  
394 488-AffiniPure goat anti-rabbit IgG and Alexa Fluor® 594-AffiniPure rabbit anti-mouse IgG  
395 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) were used as secondary  
396 antibodies. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and  
397 observations were made under a Zeiss Axioskop fluorescence microscope (Karl Zeiss, Oberkochen,  
398 Germany) equipped with an AxioCam5MR5c and coupled to an Imaging system (AxioVision  
399 Release 4.5; Karl Zeiss).

400

#### 401 *2.12 Specimen preparation for transmission electron microscopy (TEM)*

402 Analysis by TEM was employed in order to get information on the possible ultrastructural  
403 modifications of cells treated with empty and PTX-loaded SLN. NO3 NS and AC lines were used  
404 for the investigation.

405 For transmission electron microscopy, untreated cells, cells treated with unloaded SLN15 and cells  
406 treated with 100 nM PTX-loaded SLN15 for 24 h were collected, pelleted by centrifugation and  
407 then fixed with 2.5% glutaraldehyde prepared in 0.1 M phosphate buffer at 4 °C. Samples were  
408 post-fixed with 1% osmium tetroxide for 2 h, dehydrated in a graded acetone series and embedded  
409 in the Epon 812 epoxy resin. Ultrathin sections were prepared using an ultracut microtome  
410 (Reichert-Jung, Germany), collected on copper grids, stained with both lead citrate and uranyl  
411 acetate, and then observed with a Philips CM-12 transmission electron microscope.

412

### 413 *2.13 Glioblastoma cell cytotoxicity in co-culture models*

414 In co-culture experiments, 500,000 glioblastoma cells (i.e. the U87-MG cell line and the primary  
415 human glioblastoma cells CV17 and 01010627) were added in the lower chamber of Transwells 4  
416 days after seeding hCMEC/D3 cells in the upper chamber of Transwells. After 3 days of co-culture  
417 the medium of the upper chamber was replaced with fresh medium, with or without free PTX or  
418 PTX-loaded SLN (SLN 2) at different concentration (20, 50, 100, 200, 500 nM) for 24 h, as  
419 detailed under results.

420 To measure the viability of glioblastoma cells co-cultured with hCMEC/D3 cells, extracellular  
421 medium of glioblastoma cells was collected and centrifuged at 12,000 g for 15 min to pellet cellular  
422 debris, whereas cells were washed with fresh medium, detached with trypsin/EDTA (0.05/0.02%  
423 v/v), re-suspended in 0.2 ml of 82.3 mmol/l triethanolamine phosphate-HCl (pH 7.6) and sonicated  
424 on ice with two 10 s bursts (Labsonic sonicator, 100 W). LDH activity was measured in the  
425 extracellular medium and in the cell lysate: 50 µl of supernatant from extracellular medium or 5 µl  
426 of cell lysate were incubated at 37 °C with 5 mmol/l NADH. The reaction was started by adding 20  
427 mmol/l pyruvic acid and was followed for 6 min, measuring absorbance at 340 nm with Packard

428 EL340 microplate reader (Bio-Tek Instruments, Winooski, VT). The reaction kinetics was linear  
429 throughout the time of measurement. Both intracellular and extracellular enzyme activity was  
430 expressed in  $\mu\text{mol NADH oxidized/min/dish}$ , then extracellular LDH activity was calculated as  
431 percentage of the total LDH activity in the dish.

432 The level of significance was determined by a Student's  $t$  test. Statistical significance was defined  
433 as  $p < 0.05$ ,  $p < 0.02$ ,  $p < 0.01$ ,  $p < 0.005$  or  $p < 0.001$ .

### 434 3. Results and discussion

435 As described in the introduction chapter, BBB is a physical barrier characterized by tight  
436 intracellular junctions and by the absence of fenestrations that limit permeability for therapeutic  
437 molecules with no exception for molecules in the glioblastoma treatment. The use of drug-loaded  
438 nanoparticles could be a winning strategy if an active targeting and a suitable drug concentration to  
439 the brain could be obtained, provided small-sized hydrophilic vehicles are achieved to prevent rapid  
440 degradation by reticuloendothelial system and carriers with proper superficial characteristics are  
441 coupled.

442 Moving from this background, SLN were prepared using the coacervation method which uses  
443 hydrophilic polymeric as stabilizers able to confer hydrophilic surface to SLN. Further to a  
444 cytotoxicity study against hCMEC/D3 cells relating to different fatty acids [40], BA was chosen as  
445 lipid matrix. In order to take advantages of possible interaction between BBB negative charges and  
446 surface positive-charged SLN, two positive charged molecules currently used in literature were  
447 introduced in the SLN preparation: ST [44] and GCS [36]. As antineoplastic model drug PTX was  
448 chosen, a lipophilic molecule suitable to be entrapped in SLN matrix.

449 Preliminary formulation studies (data not reported) demonstrated the need to modify the preparation  
450 procedure to obtain higher drug EE% by introducing PTX after SLN melting instead of adding it to  
451 the micellar solution; probably the high PTX lipophilicity does not allow its distribution within  
452 micelles, while promoting its solubilization in the molten lipid.

453

#### 454 3.1 SLN characterization

455 In Table 1 compositions of PTX-loaded SLN are reported. SLN composition was the result of a  
456 preliminary screening carried out in a previous work [45].

457 In Table 1 ST-SLN and GCS-SLN compositions are also reported. Different formulations were  
458 obtained varying the amount of PVA 9000, ST, GCS or PTX in order to obtain the most suitable  
459 PTX vehicle as concerns Zeta potential, mean sizes, EE%, over time stability. In order to improve

SLN stability, CHOL was also added. As reported in literature [46] high CHOL/lipid ratio matrix reduced mean diameters of SLN prepared from emulsion. This led to a rigid surfactant layer with decrement in van der Waals attraction and promotion of net repulsion forces between oil droplets; therefore, agglomeration and coalescence of small oil droplets were reduced. In addition, the interaction between CHOL and surface active agent might increase the surface curvature of oil droplets consequently leading to small size of SLN after cooling down. A similar behavior can be hypothesized also in SLN obtained by coacervation. In the present experimental conditions, CHOL probably might interact with Na-BA at the interface of micelles.

In Table 1 SLN, ST-SLN and GCS-SLN mean diameter and Zeta potential are reported. Most SLN tested had sizes below 600 nm. In the neutral SLN series (SLN 0-3), increasing the amount of PTX no changes in particles dimension were noted; different behavior was noted in the case of charged SLN. In the ST-SLN series (SLN 4-10) an increase in diameter sizes can be noted when ST or PTX amounts were increased or when CHOL was introduced in the formulation. Nevertheless the presence of CHOL was essential to SLN stability: in the absence of CHOL, ST-SLN and GCS-SLN showed a sudden increase in mean size immediately after preparation. Instead, in the GCS-SLN (SLN 11-17) series a slightly decrease in SLN size was obtained increasing the GCS amount and a more marked increase was noted when a great amount of stabilizer (PVA) was used. Also in this case the presence of CHOL was necessary to formulations stability; the addition of this component led to a decrease in mean diameter and the smallest SLN were obtained using 50 mg (1% w/w) PVA (SLN 17).

The dissimilar influence of ST and GCS on ST-SLN and GCS-SLN sizes might be due to different positioning of both charged molecules: probably, lipophilic ST chain was incorporated in the lipid matrix while GCS was mainly adsorbed to the external surface of nanoparticles.

Size measurements were employed also diluting the samples with growth medium used in the in vitro experiments in order to verify SLN sizes when they are tested in vitro. The growth medium does not seem to influence SLN dimensions: all SLN have maintained their size (data not reported).

486 In Figures 1a and 1b TEM PTX-SLN2 images at different magnifications were reported. The SLN  
487 have spherical shape and sizes between 300 and 500 nm. The drug introduction does not seem to  
488 affect shape and size (data not reported). Similar characteristics were obtained also with SLN9 and  
489 SLN15.

490 The observation of Cou-6-SLN nanosuspensions under optical microscope confirmed both the  
491 spherical shape and the dimensional range of the particles. The use of fluorescent light allowed to  
492 locate Cou-6 in SLN nanosuspensions: homogeneous dispersions of fluorescent nanoparticles were  
493 observed with all formulations (data not shown).

494 In Table 1 also Zeta potential values are reported. All GC or ST-containing formulations showed  
495 positive charge with values in the 7.6 - 23.8 mV range. ST-SLN presented Zeta potential ranging  
496 from 7 to 15 mV (depending on ST amount used); these values are smaller than those of GCS-SLN  
497 and those of SLN containing the same ST percentages but prepared with methods other than  
498 coacervation [41]; probably PVA 9000 used as stabilizer locates itself externally thus partially  
499 screening the ST positive charge.

500 In GCS-SLN series, Zeta potential values confirmed the external positioning of the polymer:  
501 probably positively-charged GCS interacted with carboxylate groups on SLN surface, conferring  
502 them a positive charge. The values were influenced by GCS amount: by increasing the polymer an  
503 increase of Zeta potential from 16.9 mV to 23.8 mV was noted; the presence of CHOL and the PTX  
504 amount variation did not seem to influence this parameter.

505 In Table 2 the amount of PTX in SLN is expressed as EE% and as loading (mg PTX/mg BA).  
506 Almost all tested SLN showed a good PTX loading capacity; in particular, in neutral SLN series  
507 (SLN 0-3) PTX EE% did not significantly vary increasing the drug amount, while drug loading was  
508 increased over twice folds. In the ST-SLN series (SLN 4-10), PTX EE% was significantly  
509 influenced by several factors. Increasing ST amounts determined a reduction of PTX EE% (SLN 5)  
510 which was partially counteracted by the presence of CHOL (SLN 6) probably as a consequence of a



511 hardened structure of SLN surface. Also PVA amount seemed to be relevant in influencing PTX  
512 EE%, as higher PVA concentrations determined a reduction in PTX EE%.

513 In GCS-SLN series (SLN 11-17), EE% and PTX loading ranged from 24% to 83% and from 1.4 to  
514 5.0 mg/mg BA respectively. Also in this series PTX EE% was strongly influenced by PVA 9000  
515 amount: when the highest stabilizer amount was used (SLN 14), a dramatic decrease in drug EE%  
516 up to 24% can be noted; probably PVA 9000 formed micelles in aqueous phase able to solubilize  
517 PTX. CHOL introduction enhanced PTX EE% from 52% to 75% particularly in SLN prepared with  
518 50 mg PVA 9000. On the contrary, GCS amount variations did not seem to affect drug EE%.

519 To mime SLN behavior after administration when they are diluted in physiological fluids, drug  
520 EE% was determined also after 1:100 dilutions. When water was used as dilution medium, EE%  
521 remained almost unvaried in all ST-SLN ( $\pm 1\%$ ), except in SLN 15 in which a 10% decrease was  
522 registered. Probably, in GCS-SLN, PTX is partially trapped in GCS chains located on SLN surface  
523 and therefore it might be released upon high dilutions. To evaluate the influence of different  
524 dilution media, SLN 9, which were chosen as those having the best properties for our experimental  
525 purposes, were diluted also by pH 7.4 PBS and pH 5.5 citric buffer. No significant variation was  
526 noted upon dilution by pH 7.4 PBS ( $\pm 1\%$ ), while a certain PTX amount was released when 0.1  
527 citric pH 5.5 buffer was used, that mimed the lysosomal environment: the EE% decreased from  
528 92% to 77% and this could be due to a PTX slow release.

529

### 530 *3.2 SLN and PTX stability*

531 The studies were carried out only on SLN giving the best results concerning sizes and EE%. The  
532 chosen formulations were analyzed to verify their stability overtime. The results are reported in  
533 Table 3. SLN 9 showed a great increase in mean diameter (up to 1500 nm) after 30 days, while only  
534 a slight decrease occurred in EE%. The poor dimensional stability of SLN 9 could be due to their  
535 low Zeta potential (about 9 mV) which is probably not sufficient to prevent SLN aggregation. On  
536 the other hand, GCS-SLN (SLN 15, 16, 17) showed a higher dimensional stability although a

gradual increase in mean diameter and a gradual decrease in Zeta potential were registered due probably to a slow solubilization of the external GCS coating in the aqueous phase.

To overcome the poor stability of SLN suspension and to find an optimal storage method, they were submitted to freeze drying. In Table 4 SLN sizes after freeze-drying were reported. The freeze-drying process was carried out without adding any cryoprotectant; in fact in a previous work [45] PVA 9000 was found to be able to protect SLN during freeze-drying as well as 2% threalose.

The characterization of resulting SLN suspensions showed a mean diameter increase of all SLN analyzed probably due to a partial SLN aggregation. This increase was about 300 nm in SLN with ST while only a slight variation was registered in SLN with GCS; probably also GCS, positioning externally, could behave as cryoprotectant preventing nanoparticles aggregation. The smallest increase (about 40 nm) was noted when higher amounts of stabilizer were used. EE% was kept constant in almost all preparations; an increase in EE% (from 75% up to 89%) was registered in SLN 17 that presented a higher amount of free PTX: probably during freeze-drying a part of free PTX was internalized or absorbed onto nanoparticles.

551

### 3.3 Determination of PTX release

In Figure 2 PTX release plots from SLN using the dialysis method were reported. In this experiment drug diffusion through the dialysis membrane was influenced by drug release rate from SLN. As it can be seen, PTX was released very slowly from SLN: less than 1% PTX was released from SLN formulations in 12 hours while, at the same time, about 40% PTX diffused from PTX solution. This slow release could confirm PTX incorporation into SLN. From the  $K_d$  app data it could be seen as PTX release from GCS-SLN was still slower than from ST-GCS; these results could be the confirmation that GCS formed an external coating that further limited drug release.

560

### 3.4 Permeability of SLN across BBB

562 As shown in Figure 3, the permeability of free Cou-6 across the hCMEC/D3 monolayer  
563 remained very low at all the time points considered; the permeability of Cou-6-loaded SLN was  
564 similar to that of free Cou-6 after 0,5 h, but became significantly higher after 3 h and 24 h.  
565 Apparently there were no differences in the permeability of uncharged SLN (SLN 2) versus  
566 positively charged SLN (SLN 9 and SLN 15), although SLN 9 resulted the best permeable  
567 formulation after 24 h.

568

### 569 *3.5 In vitro cytotoxicity studies*

570 To assess the sensitivity of the five malignant glioma cell lines to PTX, we treated previously the  
571 cells with Cremophor® EL:ethanol 50:50 drug solution (free PTX) at concentrations ranging from  
572 20 nM to 1.4  $\mu$ M for 72 h.

573 After different exposure times both MTT and trypan blue assays were performed. PTX was able to  
574 block *in vitro* cellular proliferation in a dose-dependent manner; it inhibited clonogenic growth of  
575 NS (Figure 4a–4d) and hindered adhesion process in AC (Figure 4e–4i).

576 The growth inhibitory effect was more evident on AC than on NS.

577 The IC<sub>50</sub> values of free PTX at 72 h for the three NS cell lines studied were < 20 nM; for the two  
578 AC lines were < 15 nM (Figure 5). Consequently, drug concentration of 100 nM was employed for  
579 the following experiments with PTX incorporated in SLN. At this dosage, a statistically significant  
580 decrease of cell viability was caused by PTX compared with untreated cells, both as free drug and  
581 as SLN-carried, and this effect increased prolonging drug exposure time from 24 to 72 h. It was  
582 observed that also PTX encapsulated in SLN caused inhibition of sphere formation in the NS  
583 cultures (Figure 4e) and interfered with cell adhesion in AC cultures (Figure 4i).

584 Graphs in Figure 7 show cell viability of NO3 line after the treatments: as regards NS, the viable  
585 cell number decreased to 60-70% after 24 h treatment with the different PTX-SLN (Figure 6a) and  
586 to 30-40% after 72 h treatment (Figure 6b). At 24 h free PTX and all PTX-SLN gave a cytotoxicity  
587 statistically different from control, while only SLN 2 produced an increase in cytotoxicity

588 significantly different from that of empty SLN 2. Prolonging the incubation time, also SLN 9 were  
589 significantly different from the corresponding empty SLN.

590 The reduction of viability of AC was about 10-15% higher than the one of NS (Figure 7). An  
591 analogous behavior of PTX-SLN 2 and PTX-SLN 9 was noted already after 24 h incubation, while  
592 after 72 h all SLN under study produced a cytotoxicities significantly different from those produced  
593 by the corresponding empty SLN. The cytotoxicity of PTX incorporated in SLN appeared similar  
594 or even slightly higher in comparison with the one of free drug solution. In particular, SLN2 seemed  
595 to enhance growth inhibitory activity of PTX on NS lines, while SLN 9 did it on AC lines.

596 The results slightly varied in the other cell lines (data not shown), but the cytotoxic activity of PTX-  
597 loaded SLN remained always equal or greater compared with free drug. SLN 2 and SLN 9 appeared  
598 as the most effective carrier formulations.

599 Control experiments demonstrated that the exposure of the cells with the unloaded SLN at the  
600 highest SLN concentration used for the study determined a mild reduction of cell viability too,  
601 mostly on AC. Unloaded SLN 2 formulation displayed the smallest cytotoxic effect, while unloaded  
602 SLN15 had a more accentuated action on cell proliferation.

603 Treatment with PTX vehicle (50:50 v/v mixture of Cremophor® EL and ethanol) at the same  
604 concentrations used for drug solutions showed a slight cytotoxicity too (data not reported).

605 Our results suggest that, first of all, incorporation of PTX in SLN did not alter its cytotoxic  
606 capability and that these carriers, thanks to a good cell membrane permeation, are able to efficiently  
607 transport the drug inside the cells. Encapsulation into SLN appeared even to enhance the growth  
608 inhibitory effect of PTX on GBM cell lines.

609 In conclusion, loading of PTX in SLN seems to sensitize cells and to decrease their resistance to the  
610 drug.

611

612 *3.6 Study of PTX-SLN cytotoxic effect by TEM*

613 The observation by TEM enabled to evaluate the ultrastructural alterations of cells after exposure to  
614 SLN. The analysis revealed that the administration of unloaded SLN 15 didn't cause modifications  
615 to the cell morphology both in NS and in AC. Cells treated with empty SLN maintained unchanged  
616 nucleus, ergastoplasma, Golgi apparatus and mitochondria (Figure 8a).  
617 On the contrary, 24 h exposure to SLN loaded with 100 nM PTX induced severe alterations of the  
618 cellular morphology that included cytoplasmic accumulation of vacuoles, loss of mitochondrial  
619 ridges and finally mitochondrial lysis (Figure 8b-8f). The vacuoles became gradually larger and  
620 confluent and in some cases they blended and formed autophagolysosomes surrounded by a  
621 membrane. Cytoplasmic rarefaction, glycogen accumulation and destruction of all organelles  
622 suggested a typical regressive apoptotic pattern. Only in some areas there was evidence of SLN  
623 residues. These results indicate an evident cell damage caused by the PTX vehicled in the SLN.

### 624 625 *3.7 DNA damage and repair response induced by PTX-SLN treatment*

626 In response to DNA damage caused by genotoxic agents, the eukaryotic cell activates a complex  
627 checkpoint/repair pathway in order to repair the lesion before replication. The signalling involves  
628 the recruitment of the sensor protein kinase ataxia telangiectasia mutated (ATM), that initiates a  
629 transduction cascade phosphorylating downstream effectors, including H2AX histone, 53 binding  
630 protein 1 (53BP1) and the checkpoint kinases 1 and 2 (Chk1 and Chk2). When checkpoints are  
631 activated, the cell cycle is halted to allow DNA repair. If the damage is too severe, repair fails and  
632 the cell enters into apoptosis [47]. Two are the major pathways used by mammalian cells to repair  
633 double strand break damage: the homologous recombination (HR), which takes place during the S  
634 and G2 phases and is driven mainly by RAD51 protein, and the non-homologous end joining  
635 (NHEJ), which occurs during G0 and G1 phases. The key NHEJ effector is DNA-dependent protein  
636 kinase (DNA-PK), which consists of a regulatory subunit (Ku70/Ku80 heterodimer) and a catalytic  
637 subunit (DNA-PKcs) [48]. In glioma cells, increased DNA damage response and repair ability  
638 contribute to determine the radio- and chemoresistant phenotype of these cells [49].

639 After glioma cell treatment for 72 h with relatively high concentration (100 nM) of free PTX we  
640 observed a moderate activation of checkpoint and repair proteins. A mild expression of all markers,  
641 was found both in NS and in AC (data not shown), indicating that this drug is able to induce  
642 genotoxic lesions.

643 Even after NO3 cell line treatment with PTX carried by SLN, we found the presence of  $\gamma$ -H2AX  
644 positive-nuclei and a moderate expression of all sensors and effectors of checkpoint/repair pathway,  
645 both in NS (Figure 9a-9g) and in AC (Figure 9l-9q), demonstrating that the encapsulated drug  
646 maintains the ability to induce a DNA strand break damage in the glioma cell lines. As already  
647 observed for the free drug, mostly on AC lines, several cells treated with PTX-SLN appeared  
648 blocked in metaphasis (Figure 9i) and presenting a strong expression of the checkpoint/repair  
649 proteins (p-ATM, p-Chk2, p-53BP1,  $\gamma$ -H2AX, DNA-PKcs). It seemed from these findings that cells  
650 try to repair the genotoxic lesions mainly through the NHEJ system. Untreated cells were negative  
651 for all markers (inserts in Figure 9a and 9i are examples for p-ATM expression).

652 After exposure of cells to unloaded SLN for 72 h we did not observe positivity of any  
653 checkpoint/repair protein (panels h and r of Figure 9 are examples for p-ATM expression).

654

### 655 *3.8 Effective cytotoxicity of paclitaxel-loaded SLN against glioblastoma cells in co-culture models.*

656 The cytotoxicity of PTX-loaded SLN on glioblastoma cells was finally compared with that of free  
657 PTX in co-culture models of hCMEC/D3 cells and glioblastoma cells. As shown in Figures 10,  
658 11 and 12, free PTX induced a dose-dependent increase of cytotoxicity in all the glioblastoma cell  
659 lines; PTX-loaded SLN followed a similar trend and showed – at the same concentration – higher  
660 efficacy than the free drug. Also the toxicity on hCMEC/D3 cells increased dose-dependently,  
661 without significant differences between free PTX and PTX-loaded SLN (Figure 13).

662 By comparing the cytotoxicity profile on glioblastoma cells and hCMEC/D3 cells, we can state that  
663 PTX-loaded SLN achieves significant toxicity against tumor cells at concentrations that are not  
664 toxic for BBB cells. To obtain the same degree of cytotoxicity on glioblastoma cells, free PTX

665 should be used at concentration that are unacceptably toxic for brain microvascular endothelial  
666 cells. The different efficacy of free versus SLN-loaded PTX can be explained by the increased  
667 permeability of the latter through the BBB monolayer. PTX is a known substrate of P-glycoprotein  
668 (Pgp) [50], which is abundant on the luminal side of BBB cells [51]; therefore, it is a poorly  
669 delivered drug into the brain parenchima. Its loading within SLN may represent a valid strategy to  
670 overcome the efflux by Pgp at BBB levels.

671 In addition, also glioblastoma has been reported to be rich of Pgp [52] and multidrug resistance  
672 related protein 1 (MRP1) [53], a second protein that actively effluxes PTX [50], limiting its  
673 intracellular accumulation and toxicity. Therefore, a lower accumulation and toxicity of paclitaxel is  
674 expected within glioblastoma cells. The loading within SLN may promote a faster uptake and/or a  
675 lower efflux by Pgp and MRP1 from glioblastoma cells as well. Therefore, the loading in SLN may  
676 represent a valid strategy to simultaneously bypass the drug efflux at BBB and glioblastoma levels.

#### 677 **4. Conclusion**

678 The fatty acid coacervation technique, a solvent free method, was used to prepare PTX-loaded SLN.

679 The use of positive-charged molecules, such as ST and GCS, allowed to obtain positive SLN with

680 sizes in the 300-600 nm range and PTX EE% in the 25-90% range.

681 Permeability studies through hCMEC/D3 cells monolayer, assumed as *in vitro* model of the BBB,

682 showed a significantly increase in the permeation of Cou-6, used as model drug, when vehicled in

683 SLN; no significantly differences in the permeability of uncharged SLN versus positively charged

684 SLN were noted after 24 h.

685 Cytotoxicity studies on NO3 glioblastoma cell line demonstrated the absence of toxicity of

686 unloaded SLN 2 and 9 and the preservation of cytotoxic activity of PTX-loaded SLN that remained

687 always equal or greater compared with free drug.

688 In co-culture experiments with hCMEC/D3 and different glioblastoma cells, it was demonstrated

689 that, when delivered in SLN, PTX increased its cytotoxicity towards glioblastoma cells, because of

690 its increased permeation through the endothelial cell monolayer.

691 According to these *in vitro* results, the positive charge did not seem essential to enhance PTX brain

692 uptake but fundamental seem to be the PTX encapsulation into SLN.

693 Further i.v. administration of PTX-loaded SLN in rats are in progress to confirm the suitability of

694 SLN in enhancing PTX overcoming of the BBB.

695

#### 696 **Acknowledgements**

697 The authors wish to acknowledge Compagnia di San Paolo, under the research project

698 “Development of solid lipid nanoparticles (SLN) as vehicles of antineoplastic drugs to improve the

699 pharmacological glioblastoma therapy” (ORTO11WINST).



700   **References**

- 701   [1] M.K. Nicholas, R.V. Lukas, S. Chmura, B. Yamini, M. Lesniak, P. Pytel, Molecular  
702   heterogeneity in glioblastoma: therapeutic opportunities and challenges, *Semin. Oncol.* 38 (2011)  
703   243–253.
- 704   [2] M. Weller, Novel diagnostic and therapeutic approaches to malignant glioma, *Swiss Med. Wkly.*  
705   141 (2011) doi:10.4414/smw.2011.13210
- 706   [3] W. Loscher, H. Potschka, Drug resistance in brain diseases and the role of drug efflux  
707   transporters, *Nat. Rev. Neurosci.*, 6 (2005), 591-602.
- 708   [4] B.T. Hawkins, T.P. Davis, The blood-brain barrier/neurovascular unit in health and disease,  
709   *Pharmacol. Rev.* 57 (2005), 173-185.
- 710   [5] N.R. Saunders, C.J. Ek, M.D. Habgood, K.M. Dziegielewska, Barriers in the brain: a  
711   renaissance?, *Trends Neurosci.* 31 (2008), 279-286.
- 712   [6] M.L. Pinzón-Daza, I. Campia, J. Kopecka, R. Garzón, D. Ghigo, C. Riganti. Nanoparticle- and  
713   liposome-carried drugs: new strategies for active targeting and drug delivery across blood-brain  
714   barrier, *Curr. Drug Metab.* 14 (2013), 625-640.
- 715   [7] M.W. Brightman, T.S. Reese, Junctions between intimately apposed cell membranes in the  
716   vertebrate brain, *J. Cell. Biol.* 40 (1969) 648–677.
- 717   [8] A. Béduneau, P. Saulnier, J. Benoit, Active targeting of brain tumors using nanocarriers,  
718   *Biomaterials* 28 (2007) 4947–4967.
- 719   [9] M.S. Lesniak, U. Upadhyay, R. Goodwin, B. Tyler, H. Brem, Local delivery of doxorubicin for  
720   the treatment of malignant brain tumors in rats, *Anticancer Res.* 25 (2005) 3825–3831.
- 721   [10] T.F. Cloughesy, K.L. Black, Pharmacological blood–brain barrier modification for selective  
722   drug delivery, *J. Neuro-Oncol.* 26 (1995) 125–132.
- 723   [11] A.A. Sinkula, S.H. Yalkowsky, Rationale for design of biologically reversible drug  
724   derivatives: prodrugs, *J. Pharm. Sci.* 64 (1975) 181–210.

725 [12] E. Garcia-Garcia, K. Andrieux, S. Gil, P. Couvreur, Colloidal carriers and blood–brain barrier  
 726 (BBB) translocation: A way to deliver drugs to the brain? *Int. J. Pharm.* 298 (2005) 274–292.

727 [13] M.I. Alam, S. Beg, A. Samad, S. Baboota, K. Kohli, J. Ali, A. Ahuja, M. Akbar, Strategy for  
 728 effective brain drug delivery, *Eur. J. Pharm. Sci.* 40 (2010) 385–403.

729 [14] J. Kreuter, Nanoparticulate systems for brain delivery of drugs, *Adv Drug Deliver Rev* 47,  
 730 (2001) 65-81.

731 [15] P.R. Lockman, J.M. Koziara, R.J. Mumper, D.D. Allen, Nanoparticle surface charges alter  
 732 blood–brain barrier integrity and permeability, *J. Drug Target.* 12 (2004) 635–641.

733 [16] K. Park, Transport across the blood–brain barrier using albumin nanoparticles, *J. Control.*  
 734 *Release* 137 (2009) 1.

735 [17] A. Mistrya, S. Stolnika, L. Illum, Nanoparticles for direct nose-to-brain delivery of drugs, *Int.*  
 736 *J. Pharm.* 379 (2009) 146–157.

737 [18] J. Kreuter, P. Ramge, V. Petrov, S. Hamm, S.E. Gelperina, B. Engelhardt, R. Alyautdin, H.  
 738 Von Briesen, D.J. Begley, Direct evidence that polysorbate-80-coated poly(butylcyanoacrylate)  
 739 nanoparticles deliver drugs to the CNS via specific mechanisms requiring prior binding of drug to  
 740 the nanoparticles, *Pharm. Res.* 20 (2003) 409–416.

741 [19] A.E. Gulyaev, S.E. Gelperina, I.N. Skidan, A.S. Antropov, G.Y. Kivman, J. Kreuter,  
 742 Significant transport of doxorubicin into the brain with polysorbate 80-coated nanoparticles, *Pharm.*  
 743 *Res.* 16 (1999) 1564–1569.

744 [20] J. Kreuter, R.N. Alyautdin, D.A. Kharkevich, A.A. Ivanov, Passage of peptides through the  
 745 blood–brain barrier with colloidal polymer particles (nanoparticles), *Brain Res.* 674 (1995) 171–  
 746 174.

747 [21] S.S. Feng, L. Mu, K.Y. Win, G. Huang, Nanoparticles of biodegradable polymers for clinical  
 748 administration of paclitaxel, *Curr. Med. Chem.* 11 (2004), 413–424.

749 [22] J.M. Koziara, P.R. Lockman, D.D. Allen, R.J. Mumper, Paclitaxel nanoparticles for the  
 750 potential treatment of brain tumors, *J. Control. Release* 99 (2004) 259–269.

751 [23] J.F. Poduslo, G.L. Curran, Polyamine modification increases the permeability of proteins at the  
 752 blood–nerve and blood–brain barriers, *J. Neurochem.* 66 (1996) 1599–1609.

753 [24] Z. Nagy, H. Peters, I. Huttner, Charge-related alterations of the cerebral endothelium, *Lab.*  
 754 *Invest.* 49 (1983) 662–671.

755 [25] J.E. Hardebo, J. Kahrstrom, Endothelial negative surface charge areas and blood–brain barrier  
 756 function, *Acta Physiol. Scand.* 125 (1985) 495–499.

757 [26] L. Fenart, A. Casanova, B. Dehouck, C. Duhem, S. Slupek, R. Cecchelli, D. Betbeder,  
 758 Evaluation of effect of charge and lipid coating on ability of 60-nm nanoparticles to cross an in  
 759 vitro model of the blood–brain barrier, *J. Pharmacol. Exp. Ther.* 291 (1999) 1017–1022.

760 [27] W. Lu, Y. Zhang, Y. Tan, K. Hu, X. Jiang, S. Fu, Cationic albumin-conjugated pegylated  
 761 nanoparticles as novel drug carrier for brain delivery, *J. Control. Release* 107 (2005) 428–448.

762 [28] P. Blasi, A. Schoubben, G. Traina, G. Manfroni, L. Barberini, P.F. Alberti, C. Cirotto, M.  
 763 Ricci, Lipid nanoparticles for brain targeting III. Long-term stability and in vivo toxicity, *Int. J.*  
 764 *Pharm.* 454 (2013) 316–323.

765 [29] M. Patel, E.B. Souto, K.K. Singh, Advances in brain drug targeting and delivery: limitations  
 766 and challenges of solid lipid nanoparticles, *Expert Opin. Drug Deliv.* 10 (2013) 889–905.

767 [30] M.J. Glantz, M.C. Chamberlain, S.M. Chang, M.D. Prados, B.F. Cole, The role of paclitaxel in  
 768 the treatment of primary and metastatic brain tumors, *Semin. Radiat. Oncol.* 9 (1999) 27–33.

769 [31] S.H. Tseng, M.S. Bobola, M.S. Berger, J.R. Silber, Characterization of paclitaxel (Taxol)  
 770 sensitivity in human glioma- and medulloblastoma-derived cell lines, *Neuro-Oncology* 1 (1999)  
 771 101–108.

772 [32] A. Desai, T. Vyas, M. Amiji, Cytotoxicity and apoptosis enhancement in brain tumor cells  
 773 upon coadministration of paclitaxel and ceramide in nanoemulsion formulations, *J. Pharm. Sci.* 97  
 774 (2008) 2745–2756.

775 [33] S. Fellner, B. Bauer, D.S. Miller, M. Schaffrik, M. Fankhaenel, T. Spruh, G. Bernhardt, C.  
 776 Graeff, L. Faerber, H. Gschaidmeier, A. Buschsuer, G. Fricker, Transport of Paclitaxel (Taxol)  
 777 across the blood–brain barrier in vitro and in vivo, *J. Clin. Invest.* 10 (2002) 1309–1317.

778 [34] P. Ma, R.J. Mumper, Paclitaxel Nano-Delivery Systems: A Comprehensive Review, *J*  
 779 *Nanomed Nanotechnol* 4 (2013) doi:10.4172/2157-7439.1000164

780 [35] L. Battaglia, M. Gallarate, R. Cavalli, M. Trotta, Solid lipid nanoparticles produced through a  
 781 coacervation method, *J. Microencapsul.* 27 (2010) 78–85.

782 [36] A. Trapani, J. Sitterberg, U. Bakowsky, T. Kissel, The potential of glycol chitosan  
 783 nanoparticles as carrier for low water soluble drugs, *Int. J. Pharm.* 375 (2009) 97–106.

784 [37] S. Mao, W. Sun, T. Kissel, Chitosan-based formulations for delivery of si-RNA and DNA  
 785 systems for protein therapeutics and antigens, *Adv. Drug Del. Rev.* 62 (2010) 12–27.

786 [38] B.B. Weksler, E.A. Subileau, N. Perrière, P. Charneau, K. Holloway, M. Leveque, H. Tricoire-  
 787 Leignel, A. Nicotra, S. Bourdoulous, P. Turowski, D.K. Male, F. Roux, J. Greenwood, I.A.  
 788 Romero, P.O. Couraud, Blood-brain barrier-specific properties of a human adult brain endothelial  
 789 cell line, *Faseb. J.* 19 (2005) 1872-1874.

790 [39] C. Riganti, I.C. Salaroglio, M.L. Pinzòn-Daza, V. Caldera, I. Campia, J. Kopecka, M. Mellai,  
 791 L. Annovazzi, P.O. Couraud, A. Bosia, D. Ghigo, D. Schiffer, Temozolomide down-regulates P-  
 792 glycoprotein in human blood-brain barrier cells by disrupting Wnt3-signalling, *Cell Mol Life Sci*  
 793 71 (2014) 499-516.

794 [40] V. Caldera, M. Mellai, L. Annovazzi, A. Piazzzi, M. Lanotte, P. Cassoni, D. Schiffer, Antigenic  
 795 and Genotypic Similarity between Primary Glioblastomas and Their Derived Neurospheres, *J.*  
 796 *Oncol.* ID 314962 (2011) doi:10.1155/2011/314962.

797 [41] M. Trotta, E. Peira, M.E. Carlotti, M. Gallarate, Deformable liposomes for dermal  
798 administration of methotrexate, *Int. J. Pharm.* 270, (2004) 119-125.

799 [42] L. Battaglia, M. Gallarate, E. Peira, D. Chirio, E. Muntoni, E. Biasibetti, M.T. Capucchio, A.  
800 Valazza, P.P. Panciani, M. Lanotte, D. Schiffer, L. Annovazzi, V. Caldera, M. Mellai, C. Riganti,  
801 Solid lipid nanoparticles for potential doxorubicin delivery in glioblastoma treatment: preliminary  
802 *in vitro* studies, *J. Pharm. Sci.* (2014) doi:10.1002/jps.24002.

803 [43] T.R. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to  
804 proliferation and cytotoxicity assay, *J. Immunol. Methods* 65 (1983) 55–63.

805 [44] D. Pandita, A. Ahuja, V. Lather, T. Dutta, T. Velpandian, R.K. Khar, Development,  
806 characterization and *in vitro* assesment of strearylamine-based lipid nanoparticles of paclitaxel.  
807 *Pharmazie* 66 (2010) 171-177.

808 [45] D. Chirio, M. Gallarate, E. Peira, L. Battaglia, L. Serpe, M. Trotta, Formulation of curcumin-  
809 loaded solid lipid nanoparticles produced by fatty acids coacervation technique, *J. Microencapsul.*  
810 28 (2011) 537-548.

811 [46] R. Asasutjarit, S. Lorenzen, S. Sirivichayakul, K. Ruxrungtham, U. Ruktanonchai, G.C.  
812 Ritthidej, Effect of solid lipid nanoparticles formulation compositions on their size, zeta potential  
813 and potential for in vitro pHIS-HIV-hugag, Transfection. *Pharm. Res.* 24 (2007) 1098-1107.

814 [47] E. Bolderson, D.J. Richard, B.B. Zhou, K.K. Khanna, Recent advances in cancer therapy  
815 targeting proteins involved in DNA double-strand break repair, *Clin. Cancer Res.* 15 (2009) 6314–  
816 6320.

817 [48] B. Pardo, B. Gómez-González, A. Aguilera, DNA repair in mammalian cells: DNA double-  
818 strand break repair: how to fix a broken relationship, *Cell. Mol. Life Sci.* 66 (2009) 1039–1056.

819 [49] T.C. Johannessen, R. Bjerkvig, B.B. Tysnes, DNA repair and cancer stem-like cells--potential  
820 partners in glioma drugresistance?, *Cancer Treat. Rev.* 34 (2008) 558–567.

821 [50] S. Agarwal, R. Sane, R. Oberoi, J.R. Ohlfest, W.F. Elmquist, Delivery of molecularly targeted  
 822 therapy to malignant glioma, a disease of the whole brain, *Expert Rev. Mol. Med.* 13:e17 (2011)  
 823 doi: 10.1017/S1462399411001888.

824 [51] L.M. Tai, A.J. Loughlin, D.K. Male, I.A. Romero, P-glycoprotein and breast cancer resistance  
 825 protein restrict apical-to-basolateral permeability of human brain endothelium to amyloid- $\beta$ , *J.*  
 826 *Cereb. Blood F. Met.* 29 (2009) 1079-1083.

827 [52] A. Salmaggi, A. Boiardi, M. Gelati, A. Russo, C. Calatozzolo, E. Ciusani, F.L. Sciacca, A.  
 828 Ottolina, E.A. Parati, C. La Porta, G. Alessandri, C. Marras, D. Croci, M. De Rossi, GBM-derived  
 829 tumorspheres identify a population of tumor stem-like cells with angiogenic potential and  
 830 enhanced multidrug resistance phenotype, *Glia* 54 (2006) 850–860.

831 [53] E. Nakai, K. Park, T. Yawata, T. Chihara, A. Kumazawa, H. Nakabayashi, K. Shimizu,  
 832 Enhanced MDR1 expression and chemoresistance of cancer stem cells derived from GBM. *Cancer*  
 833 *Invest.* 27 (2009) 901–908.

834 **FIGURE CAPTIONS**

835

836 Figure A.1: Anatomical structures composing the BBB. AJs: adherent junctions. TJs: tight  
837 junctions. ABC: ATP binding cassette transporters.

838

839 Figure 1: TEM images. a) 2200X magnification; b) 8900X magnification.

840

841 Figure 2: PTX release plots from nanoparticles using the dialysis method

842

843 Figure 3: Cou-6 permeability 0.1% dilution. \*  $p < 0.001$

844

845 Figure 4: Effect of 72 h treatment with increasing doses (100, 500, 1400 nM) of free and 100 nM  
846 SLN10-encapsulated PTX on NO3 NS (b–e) and NO3 AC (g–k) growth. Control cells (a, f) did not  
847 receive any treatment. All 200x magnification.

848

849 Figure 5: Cytotoxicity of free PTX on NS (A) and AC (B) of NO3 glioma cell line at various  
850 dosages of drug (20, 100, 500, 1400 nM) after 72 h treatment.

851

852 Figure 6: a) cell viability of NO3 NS after 24 h treatment; b) cell viability of NO3 NS after 72 h  
853 treatment +  $p < 0.05$  vs CTRL; \*  $p < 0.05$  PTX-SLN vs SLN; ++  $p < 0.01$  vs CTRL; \*\*  $p < 0.01$   
854 PTX-SLN vs SLN

855

856 Figure 7: a) cell viability of NO3 AC after 24 h treatment; b) cell viability of NO3 AC after 72 h  
857 treatment ++  $p < 0.01$  vs CTRL; \*  $p < 0.05$  PTX-SLN vs SLN;

858

859 Figure 8: TEM of NS after treatment with SLN15 at 15000 x. (a) After 24 h treatment with  
860 unloaded SLN15. (b) After 6 h treatment with 100 nM PTX-loaded SLN15 (c) After 24 h treatment  
861 with 100 nM PTX-loaded SLN15 (d) Id, large vacuoles surrounded by a membrane, (e) Id,  
862 autophagolysosomes, 15000 x. (f) Possible remnants of SLN (20000 x).

863

864 Figure 9: Expression by IF of checkpoint/repair proteins in NO3 glioma cells after 72 h treatment  
865 with 100 nM PTX-loaded SLN10: p-ATM, 200x (a),  $\gamma$ -H2AX, 200x (b), p-Chk2, 200x (c), p-  
866 53BP1, 200x (d), DNA-PKcs, 200x (e), Ku70/80, 200x (f), RAD51, 200x (g) in NS; AC arrested in  
867 metaphases, DAPI, 200x (j); p-ATM, 200x (k),  $\gamma$ -H2AX foci, 400x (i), p-Chk2, 200x (l), p-53BP1,  
868 200x (m), DNA-PKcs, 200x (n), RAD51, 200x (o) in AC; nuclei counterstained with DAPI.  
869 Negative reaction for p-ATM in untreated NS and AC, respectively (upper and lower left inserts of  
870 panels a and i) and in NS and AC treated with unloaded SLN for 72 h (h and p); nuclei  
871 counterstained DAPI, 200x.

872

873 Figure 10: cytotoxicity against CV17 cells. +  $p < 0.05$  vs CTRL; ++  $p < 0.02$  vs CTRL;  
874 +++  $p < 0.01$  vs CTRL; ++++  $p < 0.005$  vs CTRL; +++++  $p < 0.001$  vs CTRL; \*  $p < 0.05$  SLN vs  
875 PTX; \*\*  $p < 0.02$  SLN vs PTX; \*\*\*  $p < 0.001$  SLN vs PTX.

876

877 Figure 11: cytotoxicity against O625 cells. +  $p < 0.05$  vs CTRL; ++  $p < 0.02$  vs CTRL; +++  $p <$   
878 0.01 vs CTRL; ++++  $p < 0.005$  vs CTRL; +++++  $p < 0.001$  vs CTRL; \*  $p < 0.05$  SLN vs PTX.

879

880 Figure 12: cytotoxicity against U87 cells. +  $p < 0.01$  vs CTRL; ++  $p < 0.002$  vs CTRL; +++  $p <$   
881 0.005 vs CTRL; \*  $p < 0.05$  SLN vs PTX; \*\*  $p < 0.02$  SLN vs PTX; \*\*\*  $p < 0.001$  SLN vs PTX.

882

883 Figure 13: cytotoxicity against hCMEC/D3 cells. +  $p < 0.01$  vs CTRL; ++  $p < 0.005$  vs CTRL  
884 \*  $p < 0.05$  SLN vs PTX; \*\*  $p < 0.002$  SLN vs PTX.





SLN	Na-BA* (mg)	PVA 9000 (mg)	PTX (mg)	CHOL (mg)	ST (mg)	GCS (mg)	Mean diameter (nm) (PDI)	Zeta Potential (mV)
0	53	37.5	3	-	-	-	299.2 ± 0.3 (0.053)	- 3.04 ± 0.56
1	53	37.5	3	0.5	-	-	381.3 ± 20.4 (0.152)	- 3.30 ± 0.47
2	53	37.5	4.5	0.5	-	-	386.4 ± 12.3 (0.167)	- 3.34 ± 0.79
3	53	37.5	6	0.5	-	-	383.8 ± 23.2 (0.085)	- 3.41 ± 0.60
4	53	37.5	3	-	3	-	345.6 ± 9.7 (0.089)	7.85 ± 0.91
5	53	37.5	3	-	6	-	537.7 ± 13.5 (0.105)	14.37 ± 1.52
6	53	37.5	3	0.5	3	-	581.0 ± 61.1 (0.201)	8.03 ± 0.70
7	53	25	3	0.5	3	-	507.0 ± 10.5 (0.099)	10.19 ± 0.69
8	53	37.5	3	0.75	3	-	579.6 ± 7.7 (0.115)	10.40 ± 1.14
9	53	37.5	4.5	0.5	3	-	548.6 ± 20.2 (0.130)	9.07 ± 2.45
10	53	37.5	6	0.5	3		984.9 ± 93.6 (0.103)	7.65 ± 0.85
11	53	37.5	6	-	-	10	553.5 ± 5.7 (0.196)	16.90 ± 4.13
12	53	37.5	6	-	-	16	488.4 ± 19.3 (0.213)	23.79 ± 1.81
13	53	50	6	-	-	10	469.6 ± 3.8 (0.202)	15.46 ± 2.32
14	53	100	6	-	-	10	624.3 ± 16.1 (0.205)	15.64 ± 3.12
15	53	37.5	4.5	0.5	-	10	462.7 ± 32.3 (0.227)	18.21 ± 1.67
16	53	37.5	6	0.5	-	10	476.4 ± 8.4 (0.196)	20.28 ± 1.10
17	53	50	6	0.5	-	10	374.5 ± 4.8 (0.232)	19.56 ± 1.73

886

887 Table 1

888

SLN	PTX EE%	PTX LOADING ( $\mu\text{g}$ PTX/mg BA)
0	$78 \pm 4$	$47 \pm 2$
1	$85 \pm 5$	$50 \pm 3$
2	$89 \pm 3$	$80 \pm 3$
3	$90 \pm 3$	$108 \pm 4$
4	$70 \pm 6$	$42 \pm 4$
5	$25 \pm 3$	$16 \pm 2$
6	$87 \pm 4$	$52 \pm 2$
7	$98 \pm 2$	$60 \pm 1$
8	$65 \pm 6$	$40 \pm 4$
9	$92 \pm 4$	$82 \pm 3$
11	$77 \pm 2$	$92 \pm 2$
12	$81 \pm 4$	$98 \pm 5$
13	$52 \pm 5$	$62 \pm 6$
14	$24 \pm 3$	$28 \pm 3$
15	$83 \pm 4$	$74 \pm 3$
16	$83 \pm 3$	$100 \pm 4$
17	$75 \pm 5$	$90 \pm 6$

889

890 Table 2

891

		SLN 9	SLN 15	SLN 16	SLN 17
7 days	Size (nm)	682.4 ± 15.1	783.2 ± 74.3	554.2 ± 9.5	500.1 ± 6.4
	%EE	92 ± 3	81 ± 2	80 ± 2	74 ± 3
	Zeta potential (mV)	9.15 ± 0.85	13.4 ± 0.9	15.9 ± 2.8	18.3 ± 1.6
30 days	Size (nm)	1500.0 ± 21.6	799.7 ± 63.2	598.1 ± 14.6	599.2 ± 9.5
	%EE	91 ± 4	80 ± 3	79 ± 3	73 ± 2
	Zeta potential (mV)	9.05 ± 0.54	11.6 ± 1.4	14.5 ± 3.3	14.4 ± 3.1
60 days	Size (nm)	1536.8 ± 39.2	933.8 ± 17.9	669.8 ± 27.3	686.1 ± 12.4
	%EE	89 ± 4	80 ± 3	71 ± 2	73 ± 2
	Zeta potential (mV)	9.07 ± 0.65	10.7 ± 2.1	13.9 ± 3.4	12.9 ± 4.1

892

893 Table 3

894

	SLN 9	SLN 15	SLN 16	SLN 17
<b>Mean diameter (nm)</b>	842.0 ± 17.9	632.4 ± 18.4	592.3 ± 18.0	408.4 ± 5.8
<b>Zeta potential (mV)</b>	8.66 ± 1.02	18.56 ± 1.34	19.68 ± 1.98	19.87 ± 1.45
<b>PTX EE%</b>	88 ± 3	81 ± 2	83 ± 2	89 ± 3

895 Table 4

896

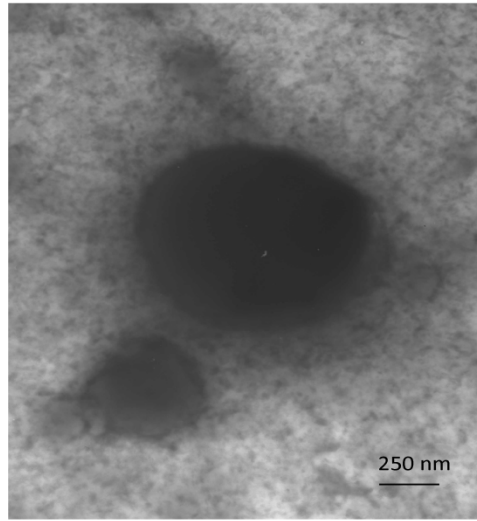
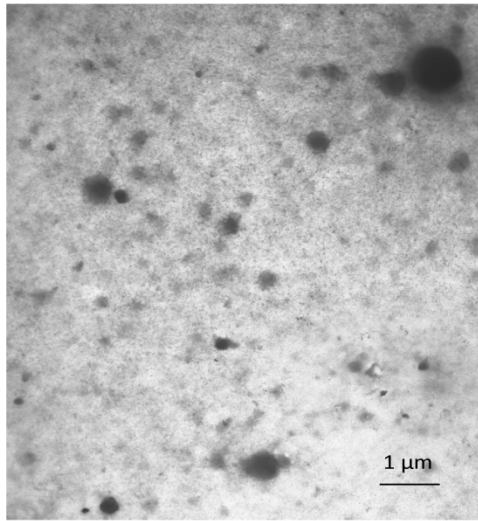


Figure 1a and 1b

Chirio et al

897

898

899

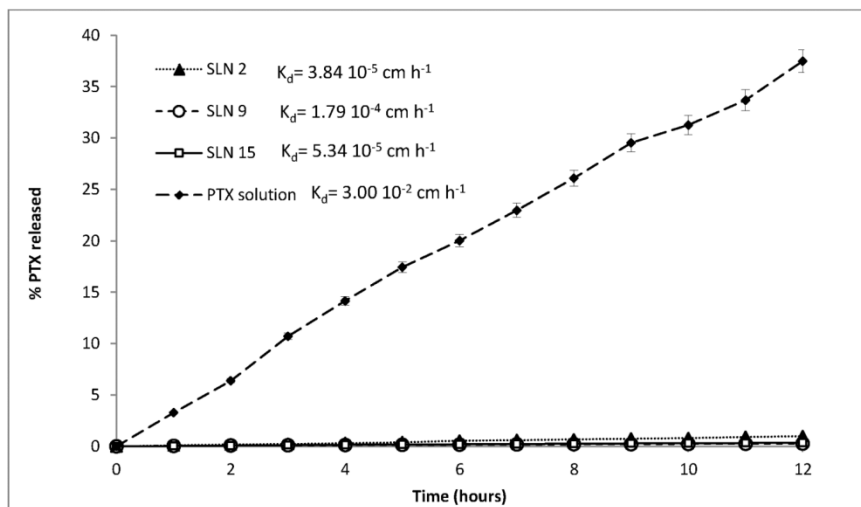


Figure 2

Chirio et al

900

901

902

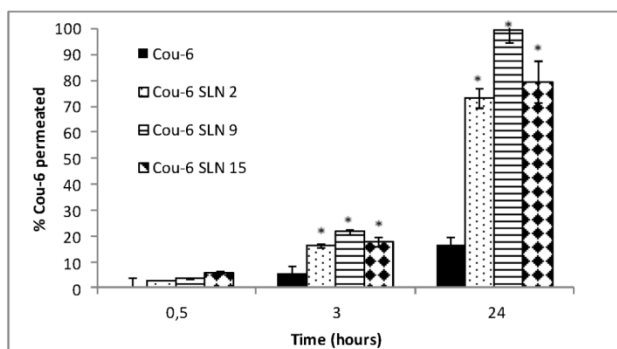


Figure 3

Chirio et al

903

904



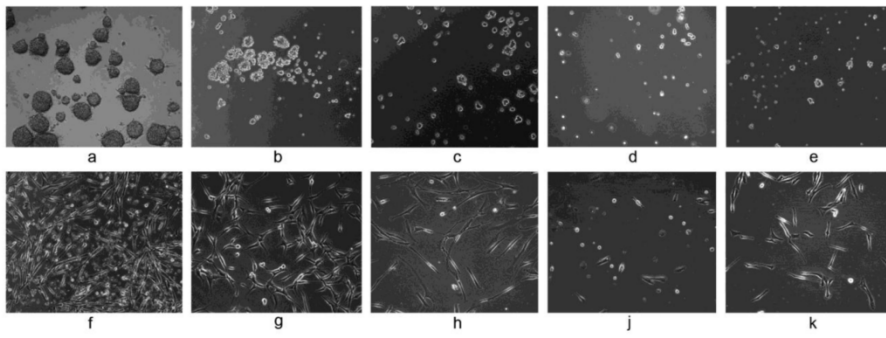


Figure 4

Chirio et al

905

906

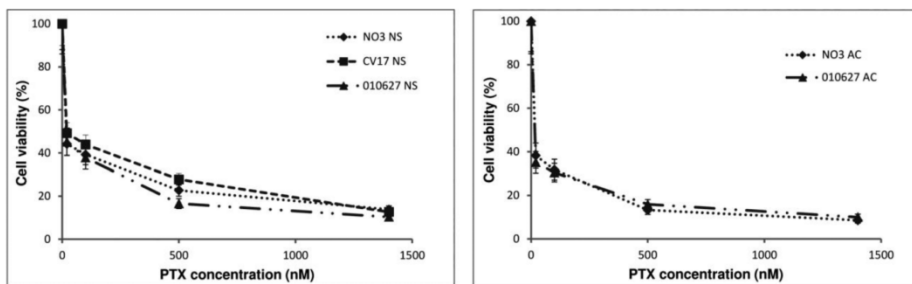


Figure 5

Chirio et al

907

908

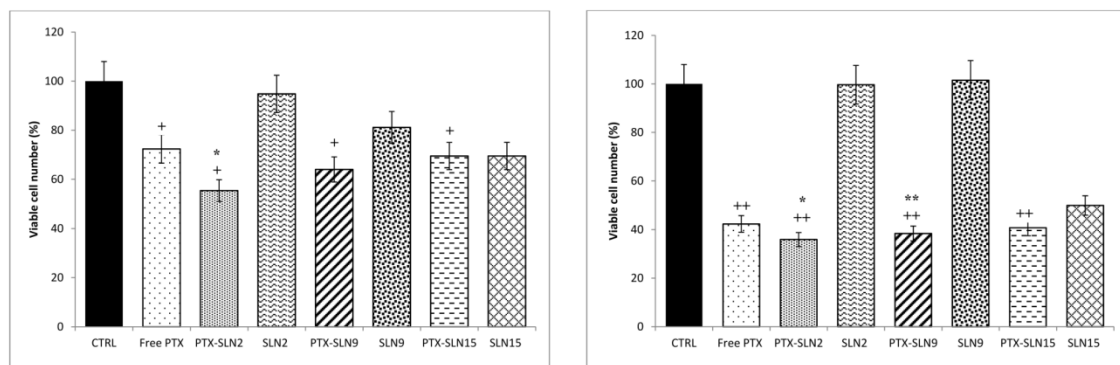


Figure 6

Chirio et al

909

910

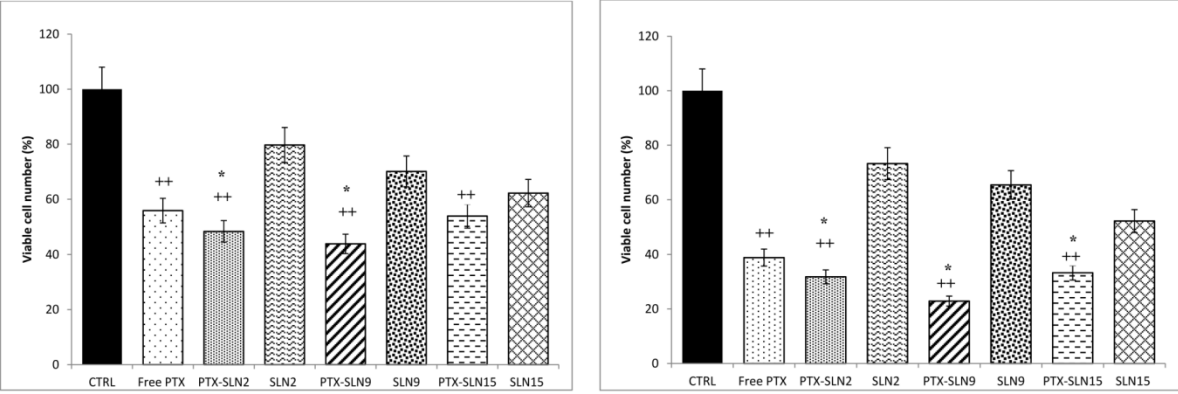


Figure 7

Chirio et al

911

912

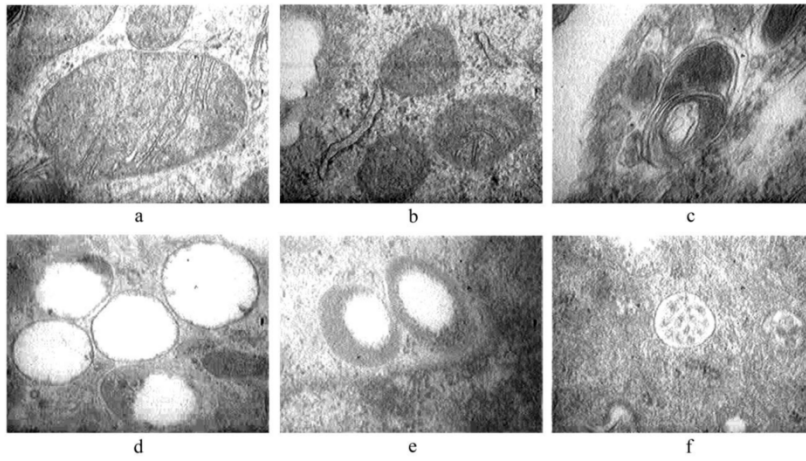


Figure 8

Chirio et al

913

914

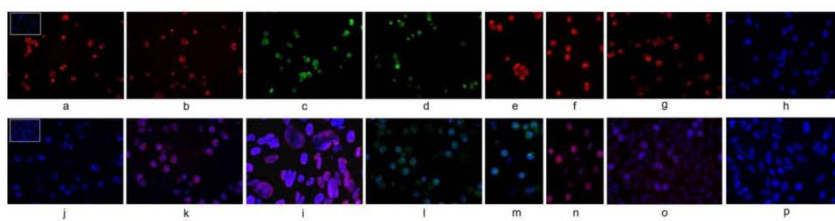


Figure 9

Chirio et al

915

916

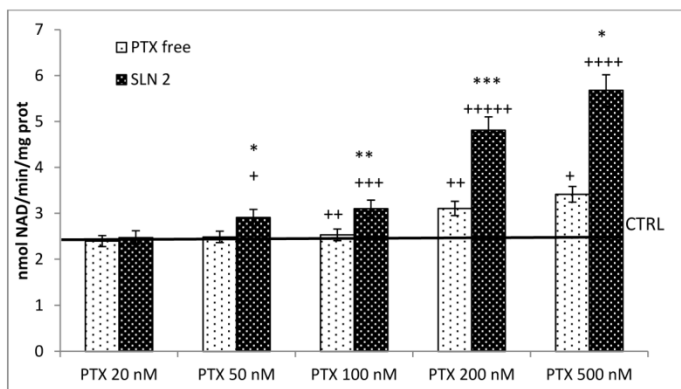


Figure 10

Chirio et al

917

918

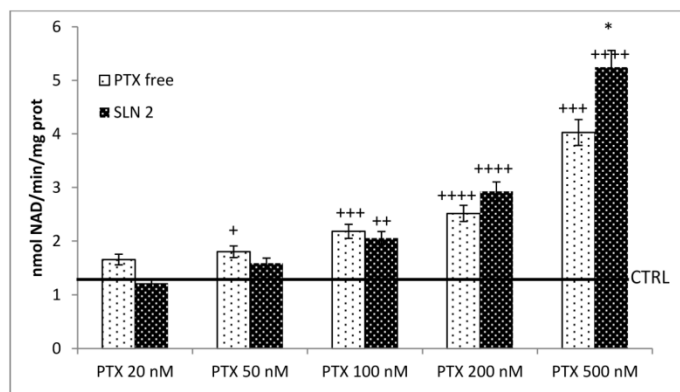


Figure 11

Chirio et al

919

920



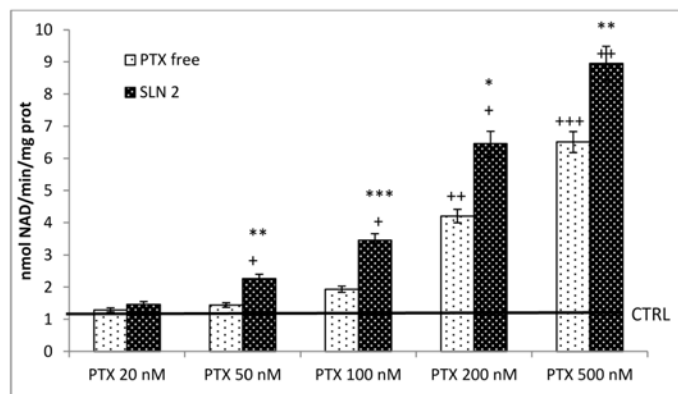


Figure 12

Chirio et al

921

922

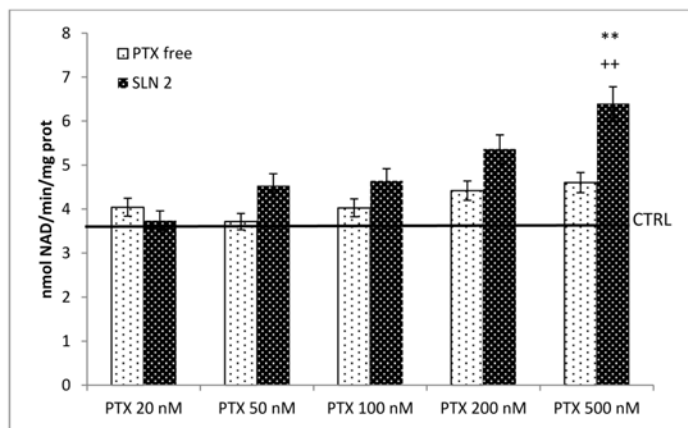


Figure 13

Chirio et al